



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification : A61K 9/127	A1	(11) International Publication Number: WO 92/10166 (43) International Publication Date: 25 June 1992 (25.06.92)
(21) International Application Number: PCT/EP91/02377 (22) International Filing Date: 9 December 1991 (09.12.91) (30) Priority data: 90810969.7 11 December 1990 (11.12.90) EP <i>(34) Countries for which the regional or international application was filed:</i> AT et al. (71) Applicant (for all designated States except US): SINTETICA S.A. [CH/CH]; CH-6850 Mendrisio (CH). (72) Inventors; and (75) Inventors/Applicants (for US only) : SCHNEIDER, Michel [FR/CH]; Domaine du Moulin, 34, route d'Annecy, CH-1256 Troinex (CH). TOURNIER, Hervé [FR/FR]; 300, rue du Riondet, F-74520 Valleiry (FR). HYACINTHE, Roland [FR/FR]; Aubonne, F-74140 Douvaine (FR). GUILLOT, Christian [FR/FR]; Les Eplanes, F-74160 Le Châble-Beaumont (FR). LAMY, Bernard [FR/CH]; 7, rue des Lilas, CH-1202 Geneva (CH).		(74) Common Representative: SCHNEIDER, Michel; Sintetica S.A., Centre de Recherche, 7, route de Drize, CH-1227 Carouge (CH). (81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KR, LU (European patent), MC (European patent), NL (European patent), SE (European patent), US. Published <i>With international search report.</i>
(54) Title: METHOD FOR MAKING LIPOSOMES OF ENHANCED ENTRAPPING CAPACITY TOWARD FOREIGN SUBSTANCES TO BE ENCAPSULATED (57) Abstract <p>Liposome vesicles are prepared containing water or very dilute solutions encapsulated therein. These "empty" liposomes are suspended in a carrier liquid containing, dispersed therein, substances of interest to be loaded into the vesicles and incubated for a period of time at temperatures above the lipids transition temperature, whereby loading by transmembrane permeation occurs in high yields.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LJ	Liechtenstein	SU ⁺	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE*	Germany	MC	Monaco	US	United States of America
DK	Denmark				

⁺ Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

**METHOD FOR MAKING LIPOSOMES OF ENHANCED
ENTRAPPING CAPACITY TOWARD FOREIGN SUBSTANCES TO BE
ENCAPSULATED**

The present invention concerns liposomes with improved trans-membrane loading capacity. It also concerns a method for making such liposomes and the loading thereof with substances of interest.

As is well known, liposomes consist of small vesicles bounded by a membrane wall of lamellar lipids surrounding a core filled with an entrapped aqueous liquid phase. Liposome vesicles can be loaded with foreign substances like drugs and may thereafter be used to selectively deliver such encapsulated drugs to selected organs in the body. Liposomes, when in suspension in aqueous carriers, are particularly suitable to deliver drugs to patients by parenteral, peroral, topical and inhalation routes. Liposome drug formulations may improve treatment efficiency, provide prolonged drug release and therapeutic activity, increase the therapeutic ratio and may reduce the overall amount of drugs needed for treating a given kind of ailment or disorder. For a review, see *Liposomes as Drug Carriers* by G. Gregoriadis, Wiley & Sons, New-York (1988).

Many methods exist for preparing liposomes and loading them with foreign substances of interest, most of which methods involve forming the liposome vesicles within an aqueous carrier liquid containing said substances distributed therein. During liposome formation, a portion of said carrier liquid becomes entrapped within the vesicles, together of course, with a small amount of the desired substances to be encapsulated. This technique is called "passive entrapment". The efficiency of loading liposomes with passively entrapped aqueous phases is often quite low because it strongly depends on the nature of the carrier phase and, particularly, the concentration of the substances dissolved therein which may affect the yield of liposome formation. However, for drug

delivery purposes, the loading efficiency (which is generally defined as the weight of material entrapped over the total weight of material involved in entrapment) is usually not critical because the non-entrapped material can generally be recovered and reused afterwards; hence, the important factor is rather the ratio of useful entrapped material versus the weight of the lipids used for entrapment, i.e., the lipids involved in forming the liposomes membrane. Clearly, minimizing lipid dead-weight upon injection or otherwise, i.e. keeping the weight of vector drug carriers administered to patients to the lowest possible level for a given amount of therapeutically active species is a strong asset in the development of new pharmaceuticals or diagnostic reagents. Now, obviously, the ratio of the weight of encapsulated material over the weight of encapsulating lipids is in direct relation with the so-called captured volume, i.e. the volume of the aqueous phase entrapped in the liposomes core per weight of liposome lipids ($\mu\text{l}/\text{mg}$).

In a classical passive entrapment method described by BANGHAM et al., (J. Mol. Biol. 12, (1965), 238), the aqueous phase containing the compound of interest is put into contact with a film of dried phospholipids deposited on the walls of a reaction vessel. Upon agitation by mechanical means, swelling of the lipids will occur and multilamellar vesicles (MLV) will form. The captured volume of MLV's is low, typically near 2 to 4 $\mu\text{l}/\text{mg}$ of lipids. By sonication, the MLV's can be converted to small unilamellar vesicles (SUV) whose captured volume is even smaller, e.g., near 0.5 - 1 $\mu\text{l}/\text{mg}$. Other methods of preparation giving liposomes with larger captured volume have been described, particularly large unilamellar vesicles (LUV). For instance, DEAMER & BANGHAM (Biochim. Biophys. Acta 443, (1976), 629) have described a method in which membrane forming lipids are dissolved in ether and, instead of first evaporating the ether to form a thin film on a surface, this film being thereafter put into contact with an aqueous phase to be encapsulated, the ether solution is directly injected into said aqueous phase and the ether is evaporated afterwards, whereby liposomes with cap-

tured volumes of 14 $\mu\text{l}/\text{mg}$ were obtained. Also the Reverse Phase Evaporation (REV) method described by SZOKA & PAPAHDJOPOULOS (P.N.A.S. 75, (1978), 4194) in which a solution of lipids in a water insoluble organic solvent is emulsified in an aqueous carrier phase and the organic solvent is subsequently removed under reduced pressure, gave liposomes with captured volumes of 8 - 15 $\mu\text{l}/\text{mg}$ of lipids.

Improved passive entrapment has been achieved by subjecting liposomes to successive dehydration and rehydration treatment, or freezing and thawing; dehydration was carried out by evaporation or freeze-drying. This technique is disclosed for example by KIRBY & GREGORIADIS (Biotechnology, November 1984, 979-984). Also, SHEW & DEAMER (Biochim. et Biophys. Acta 816 (1985), 1-8) indicate that liposomes prepared by sonication are mixed in aqueous solution with the solute to be encapsulated, and the mixture is dried under nitrogen in a rotating flask. Upon rehydration, large liposomes are produced in which a significant fraction of the solute has been encapsulated.

Further attempts to increase the amount of substance entrapped in liposomes by using higher concentrations thereof in the carrier liquid have been brought about with little success. Indeed, as said before, the captured volume often decreases at high solute concentrations in the carrier phase which indicates that the presence of the substances to be entrapped in high concentrations has a detrimental effect on captured volumes. For instance, SZOKA et al. (loc.cit.) have reported a progressive decrease in the entrapment of cytosine arabinoside with increasing concentrations of NaCl in the carrier liquid. A similar situation is described in WO-A-89/11272 (MINCHEY et al.) according to which a drastic decrease in cephalosporin entrapment yield occurs with increasing the drug concentration in the carrier liquid.

According to another route for filling liposomes with foreign non-lipidic substances, conditions are provided under which such substances can penetrate into the vesicle core through its walls; this technique, called "transmembrane loading", involves internalizing the substances to be en-

capsulated into the liposome vesicles after the latter have been formed. Normally, the crossing over of the lipid membrane by foreign substances (particularly ionic) is difficult because the incoming substances are repelled by the polar groups of said lipids. However this effect can be minimized by incorporating "shield" carriers to the lipid membrane. For instance, liposomes can be loaded with cations at room temperature when the lipid membrane contains a lipophilic carrier such as acetylacetone (BEAUMIER et al., J. Nucl. Med. 32 (1982) 810). Otherwise, foreign substances may be internalized into liposomes by osmotically controlled permeation through the lipidic membrane wall. For instance, the uptake of foreign substances by the liposomes can be promoted by a transmembrane ionic gradient, e.g. a Na^+/K^+ gradient as disclosed in J. Biol. Chem. 260 (1985), 802-808. A pH gradient is also effective for promoting transmembrane loading as mentioned in Biochim. Biophys. Acta 857 (1986), 123-126, WO-A-89/04656 and PCT/US85/01501. However, this technique is limited to some specific categories of drugs, more particularly weak bases, as acknowledged in Chem. Phys. Lipids 53 (1990), 37. Furthermore, making liposomes in a carrier phase of pH different from that of the core phase is difficult and, in addition, too low or too high a pH may cause membrane damage due to premature hydrolysis of the lipids.

In EP-A-361.894 (THE HEBREW UNIVERSITY), there is disclosed a technique in which amphipatic drugs are loaded into liposomic vesicles by transmembrane internalization under the control of a post-generated pH gradient. The key feature of this technique depends on the leakage of ammonia (NH_3) from the core of liposome vesicles loaded with an aqueous solution of an ammonium compound and placed in an ammonium-free carrier medium. Leakage of NH_3 from NH_4^+ releases a proton with consecutive lowering of the pH of the entrapped liquid and consecutive establishment of a pH gradient across the liposome membrane, i.e. the carrier liquid becomes alkaline relative to the internal content of the liposome core. When an amphipatic compound (e.g. a drug with a deprotonated amine group) is added to the "alkalinized" carrier liquid, the

system will tend to reequilibrate and a diffusion of said amphipatic compound into the core of the liposomes through the lipid membrane will occur.

Techniques in which dehydrated and rehydrated liposomes are subjected to transmembrane loading also exist. For example, US-A-4,673,567 (SHIONOGI & Co.) discloses preparing "empty" MLV liposomes in an ion-free aqueous carrier liquid and dehydrating these liposomes by lyophilization; then the dried liposomes are rehydrated by suspending in a carrier liquid containing a drug like Fluorouracil, Cefalexin or the like, and incubation is carried out by heating for 5 min at 50°C, whereby a significant portion of the drug dissolved in the carrier liquid becomes entrapped in the liposomes. The rationale behind this approach is that "freeze-drying liposomes produces structural defects in the bilayer membrane and heating above the transition temperature removes these defects" as acknowledged in an article by H. JIZOMOTO et al. in Chem. Pharm. Bull. 37 (1989), 3066-3069. However, as indicated in US-A-4,673,567, this method is hampered by a considerable reduction in the captured volume when the carrier liquid contains ionic solutes. For instance, from the data reported in Table 1, col.3 of this document, when using isotonic brine or .02 phosphate buffer as the carrier liquid, the transmembrane drug take-up was practically negligible, whereas when the drug was dissolved in pure water a value of captured volume of 16.6 µl/mg of lipid was reported. Furthermore, it should be realized that in current practice, high values of captured volumes are not easily attainable. For instance, in a recent survey article: "The accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient", by T.D. MADDEN & al., in Chemistry and Physics of Lipids 53 (1990), 37-46, the quoted captured value does not exceed about 1 - 2 µl/mg of phosphatidylcholine.

It can be seen from the foregoing brief summary that the techniques of the prior art for loading liposomes are complicated, expensive and not generally applicable to all types of drugs and media administrable via liposomes, namely ionic

species are generally difficult to entrap. It was therefore the aim of the present inventors to increase the captured volume significantly, although avoiding tedious and expensive pretreatments of the film forming lipids (e.g. lyophilization as taught by H. JIZOMOTO in Chem. Pharm. Bull. 37 (1989), 1895-1898) and, simultaneously, efficiently condition the membrane forming lipids for enhancing the transmembrane loading capacity toward substantially all kinds of solutes in aqueous media including ionic species. This has now been accomplished by embodying the method disclosed in the annexed claims which appears to be based on an osmotically controlled permeation process.

In brief, in the present invention, one prepares liposomes by any available method, said liposomes, as made, being "empty". By "empty" liposomes, one wishes to say that the aqueous phase entrapped therein is only pure water or, otherwise, is made of very dilute solutions of non-ionic substances or electrolytes. Generally speaking, if solutes are present in the entrapped phase of the newly prepared "empty" liposomes, the osmolality thereof should not exceed about 0.2 Osm/kg; if the solutes are electrolytes, the ionic strength of the entrapped liquid should not exceed about 0.1. Then, once the so-called "empty" liposomes have been made, they are suspended in a carrier liquid containing one or more substances of interest to be encapsulated, and one proceeds to incubate the system at a temperature above the transition temperature T_C for a time sufficient to ensure efficient vesicle loading by transmembrane permeation.

It should be stressed at this stage that one of the chief factors in this invention relates to the absence, or the presence in only minute quantities, of solutes in the aqueous phase where the liposomes are initially prepared, particularly when the solutes are electrolytes. In this connection, it has been noted that the "quality" of the liposome vesicles, that is the extent of their ability to generate as high as possible a captured volume, strongly depends on the ionic strength of this aqueous liquid phase; naturally, this aqueous phase is also that one which is trapped within the core

of the nascent "empty" liposomes at the time when they form. This situation contrasts strongly with the teaching of the prior art (e.g. US-A-4,673,567) where the captured volume is affected only by ions present outside the liposome vesicles, i.e., the ions within the carrier liquid in which the liposomes are incubated after dehydration. Hence the the fundamental unexpected feature now discovered by the present inventors is that the transient permeability of the membrane of the newly prepared liposomes depends mainly on the nature of the liquid in which the "empty" liposomes have been prepared initially, not on the liquid used for the post-incubation treatment. Actually, there is an inverse relationship between the concentration of electrolyte in the liquid used for making the empty liposomes and the captured volume of foreign substances encapsulated subsequently. The leaner this liquid, the higher the captured volume.

For instance, in one embodiment of the invention, membrane-forming lipids are admixed with an aqueous liquid carrier, for instance water at pH of 1 to 12, but preferably around neutrality, optionally containing diluted buffers and/or non-ionic stabilizers such as sugars or other polyols, and the carrier is maintained for a period of time at a temperature of a few degrees C above the crystal/liquid transition temperature (T_C) of the hydrated lipid, this being preferably within a narrow range of temperature values. The span of this range can be of about 20°C, and the most preferred temperature is in the vicinity of the mid-point of this range, i.e. about 4 to 10°C above T_C . The time required to effect efficient hydration and conditioning of the lipids corresponds to the time required to obtain a homogeneous solution or dispersion thereof in the carrier phase, agitation being optional. Generally, a gentle swirl of the liquid is sufficient to ensure homogenization, but faster stirring is also possible if desired. It should be noted that the average size of the liposomes which form during hydration and conditioning of the lipids may depend on the rate and the mode of agitation. Generally, very slow agitation leads to liposomes of larger average size and internal capacity than when oper-

ating under more violent agitation. It should also be noted that all preliminary treatments of the lipids recommended in the prior art to increase the loading capacity of the liposomes, i.e. freeze-drying, thawing, evaporating from a solution into thin films on the walls of a laboratory flask, and other alike pretreatments, although harmless, are absolutely unnecessary in the method of the present invention; however, since these preliminary treatments are not harmful, they can be performed if desired.

Irrespective of the ionically charged component, the lipids or mixture of lipids to be used in the present invention substantially include all compounds commonly used in the field of liposomes, i.e. glycerophospholipids, non-phosphorylated glycerides, glycolipids, sterols and other additives intended to impart modified properties to liposomic membranes. Preferably, they comprise at least a polarizable component (even in minor quantity), namely a cationic or anionic function carrying lipid or an ionizable tenside such as a fatty alcohol diphosphate ester, e.g. dicetyl phosphate (DCP) or a higher alkyl amine like stearylamine (SA). Charged phospholipids, i.e. fatty acid glycerides phosphatides like phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidyl-inositol (PI), phosphatidyl-serine (PS) from natural sources or synthetic (such as dipalmitoyl-phosphatidic acid (DPPA), dipalmitoyl-phosphatidyl glycerol (DPPG), etc.) are convenient polarizable lipid components. The glycerophospholipids may include for instance the following synthetic compounds: Dipalmitoyl-phosphatidyl-choline (DPPC), dipalmitoyl-phosphatidyl-ethanolamine (DPPE) and the corresponding distearoyl- and dimyristyl- phosphatidyl-choline and -ethanolamine (DSPC; DSPE; DMPC and DMPE). Phospholipids may also include natural phospholipids which have been subjected to more or less extensive hydrogenation, for instance egg and soy phosphatidylcholine.

The glycolipids may include cerebrosides, galactocerebrosides, glucocerebrosides, sphingomyelins, sulfatides and sphingolipids derivatized with mono-, di- and trihexosides. The sterols, which should be used with parsimony, as too much may impede membrane permeation, encompass cholesterol, ergosterol,

coprostanol, cholesterol esters such as hemisuccinate (CHS), tocopherol esters and the like.

In order to perform the method of the invention, a proportion of lipid or mixture of lipids, with or without additives, is admixed into a volume of non-ionic aqueous liquid (or an aqueous liquid whose ionic strength does not exceed .1 and the osmolality of which does not exceed .2 Osm/kg), and hydration of the lipids is allowed to proceed until the mixture is homogeneous, by which time the desired liposome vesicles will have been formed. When the liquid phase is essentially water, the relative proportions of the lipids and the aqueous liquid are not critical but, evidently, a minimum of liquid is necessary to ensure correct a dispersion of the lipids therein. Normally, for 1 part by weight of lipids or mixtures of lipids and membrane forming additives, there is used at least 20 parts of liquid phase. Excellent results are however observed with smaller lipid to liquid weight ratios, e.g. in the order of 0.1 to 1%. If the aqueous liquid used to make the empty liposomes is thereafter used as the carrier phase for incubation (i.e. the substance to be encapsulated is simply added to the liquid in which the liposomes have been made), it is obviously preferable that the amount of the liquid relative to the lipids be not too great, as this would lead to useless dilution of the substance to be encapsulated and lower entrapping yields. Notwithstanding, in case the liposome dispersion is too dilute, concentration of the vesicles can be brought about by centrifugation (in the range of 10^3 to 10^5 g), or by partial evaporation of the liquid, or by ultrafiltration through suitably calibrated semi-permeable membranes.

After the lipids have been added to the liquid phase, the system is allowed to homogenize upon standing with occasional shaking or under more constant agitation. The temperature at which this operation is brought about has been defined already before. The liquid may be raised to the desired temperature before or after adding the lipids. The preferred temperature will naturally depend on the kind of lipids or mixture of lipids used; however for the most commonly used

lipids and lipid mixtures, the hydration and homogenization temperature will be selected in a range from about 40°C to 80°C.

The compositions of the liquid phases in which the liposome vesicles are generated to be embodied in this invention are very many. Besides pure water, solutions of diluted electrolytes like mineral salts, or of non-ionic species such as glycols or polyols stabilizers can be used. For example, the following non-ionic stabilizers can be mentioned: sugars like sucrose, glucose lactose and maltose, polyols like glycols, glycerol, sorbitol, mannitol, polyethylene-glycol, dextran, xanthan and the like.

The time necessary to achieve hydration and conditioning of the lipids into liposomic vesicles of outstanding encapsulating properties may vary from a few minutes to several hours at the desired temperature, but heating times not exceeding about 30 to 60 min are generally preferred.

Naturally, the method of the invention also applies to initial conditions for contacting the lipids and the aqueous carrier other than merely admixing the components together. For instance, as said before, other routes for making liposomes can be applied as well, e.g., first forming a lipid film on surfaces (like that of a round bottom flask, or of glass beads, or the interstitial surface of a bundle of wirelike materials) and then contacting or circulating an aqueous phase on said lipid film until hydration of the latter becomes effective. If desired, hydration with sonication can be effected. It has however been observed that the simplest preparation embodiments of the present invention lead to liposomes with the highest entrapping capacity.

The liposomic vesicles obtained according to the invention are generally in a range of sizes of about 80 nm to about 5 μ m, sizes in the vicinity of 300 to 2000 nm being preferred when therapeutic or diagnostic applications by injection are considered. These liposomes are preferably of the MLV type, but other kinds of liposomes can also be made depending on the choice of operational parameters. The size distribution of these liposomes is usually rather wide but,

if narrower size distributions are desired, calibration techniques such as filtration under pressure or extrusion through microporous membrane can be successfully applied. It should be noted in this connection that the calibration of empty liposome versus that of loaded liposomes is advantageous because no substance being entrapped in the liposomes yet, no leakage thereof can occur during extrusion. Also extrusion of empty liposomes is easy because of their inherent low viscosity. Hence extrusion is preferably performed below T_c , e.g. at room temperature, or below, which provides optimal entrapment yields (high captured volumes) in the subsequent transmembrane loading steps. Furthermore, in the present invention, the type, size and concentration of the empty liposomes can be adapted to the needs before incubation, no loss of entrapped substance being involved in these operations.

However, the fundamental advantage of the liposomes obtained in the present invention relates to their surprising loading capability toward most foreign substances to be encapsulated. This loading can be easily performed by simply incorporating the substance to be encapsulated in the liquid in which the liposomes have been formed or in another liquid carrier in which the empty liposomes are subsequently suspended, this liquid serving as the carrier phase for incubation with the substances to be encapsulated. The substances to be encapsulated are being brought either neat or in the form of solutions. Then, incubation of the system is carried out for a period of time, at a temperature above the lipid transition temperature T_c . When the substance to be encapsulated is used neat, it will first dissolve in the liquid serving as carrier and from there it will permeate through the membrane and penetrate into the liposome core. A similar process will occur if the foreign substance is added in solution; here, the incubation carrier liquid will first become diluted by said solution and the dissolved substance will then penetrate into the liposomes as mentioned before. It is particularly interesting to note that, in contrast with the prior art, the vesicle transmembrane loading mechanism inherent to the present invention occurs satisfactorily even when

ions are present in the carrier liquid used for incubation, said ions being either constituents of the incubation carrier itself (buffers or saline) or of the substances of interest to be encapsulated. It appears that when the water initially captured by the liposome vesicles is either pure or contains substances in low concentration, the inhibition to permeation noted in the prior art is overcome. What is particularly surprising in this invention is once a portion of an ionic substance has permeated the membrane during incubation, it does not inhibit the penetration of the remaining portion still in the carrier liquid.

The time of incubation may vary in function to the rates of permeation into lipids typical of the substances to be encapsulated, the nature and concentrations of the liposomes in the carrier phase, and the temperature of incubation. The factor that will generally determine the end of the incubation time is the condition where the concentrations of the encapsulated substances are the same inside and outside the liposomes. At this moment, equilibrium has been reached and prolonging incubation has no further purpose. Of course, the higher the temperature, the faster equilibrium is established; however too high temperatures may be detrimental to the liposome properties, namely to the specific encapsulation capacity, i.e., the ratio of core volume to weight of lipids; hence the incubation temperatures may range from about T_c to about 150-200°C, the preferred range being from about 40 to 130°C. It should be noted in this connection that if the incubation temperature is in the high portion of the given range, say, 100 to 150°C, substantial sterilization of the liposomes will occur simultaneously with incubation. Alternatively, one may effect sterilization and incubation independently and subsequently. The heating means to bring the liposomes and the products to be encapsulated to incubation temperatures are conventional in the field and naturally include microwave heating means. It should however be remarked that the temperature of initially hydrating and conditioning the lipids for liposome formation is not to be confused with the incubation temperature, although both can be

identical in some embodiments. Actually, the hydration temperature range is much narrower than the incubation temperature range; if hydrating and conditioning were carried out outside the given range, say at about 80°C with lipids or mixtures thereof having a T_C around 45 - 55°C, liposomes of inferior quality would be obtained, i.e., with lower entrapment capacity and lower volume to weight ratio.

It should be noted that one further advantage of the present invention is that the concentration of lipids in the aqueous carrier used for incubation has no significant influence on the internalization capacity and efficiency of the liposomes toward foreign substances added to said aqueous carrier. Hence by concentrating the liposomes in the aqueous carrier, i.e. by increasing the lipid to carrier weight ratio, one may favorably influence the entrapment yield and reduce the amount of residual non entrapped substance to be recovered and reused afterwards. This can be illustrated by remarking that the ultimate concentration of the foreign substances in the liposome core only depends on the initial concentration thereof in the incubation carrier liquid, not on the total weight of the foreign substances used for encapsulation. Hence this total weight can be reduced for a given concentration by decreasing the amount of liquid used for incubation and, conversely, increasing the concentration of lipids in the carrier phase will lead to an increase of entrapment yield.

The substances to be entrapped in the liposomes according to the invention include any imaginable therapeutically or diagnostically active compounds. As such, one may recite drugs like analgesics, narcotics, antibiotics, sulfamides, steroids, X-ray opacifiers, NMR contrast agents and the like. X-ray opacifiers include for instance organic iodinated compounds like N,N'-bis[2-hydroxy-1-(hydroxymethyl)-ethyl]-5-[(2-hydroxy-1-oxopropyl)-amino]-2,4,6-triiodo-1,3-benzene-dicarboxamide (iopamidol); metrizamide; diatrizoic acid; sodium diatrizoate; meglumine diatrizoate; acetrizic acid and its soluble salts; diprotrizic acid; iodamide; sodium iodipamide; meglumine diopamide; iodohippuric acid and the

soluble salts thereof; iodomethamic acid; iodopyracetiodo-2-pyridone-N-acetic acid; 3,5-diiodo-4-pyridone-N-acetic acid (Iodopyracet) and its diethyl ammonium salt; iothalmic acid; metrizoic acid and its salts; the ipanoic, iocetamic and iophenoxic acids and their salts; sodium tyropanoate; sodium opidate and other like iodised compounds.

The following examples illustrate the invention:

Example 1

Thirty g of phospholipids (a 9/1 molar ratio of hydrogenated soy lecithin (Phospholipon 100H from NATTERMANN-PHOSPHOLIPID GmbH, Köln, Germany) and dipalmitoylphosphatidic acid disodium salt (DPPA) with a trace amount of ^{14}C -labeled tripalmitin (Amersham) in solution in chloroform (250 ml) were introduced in a 10 l reaction flask. After evaporation of the chloroform under reduced pressure, there were added 6 l of distilled water at 55-60°C (the transition temperature of the hydrated lipids used was 54°C as determined by differential scanning calorimetry) and the solid lipids were allowed to hydrate and distribute homogeneously through the liquid with occasional gentle shaking, whereby liposomes of the MLV type did form in high yield. After about 1 hour, the liposome suspension containing 5 mg/ml of lipids was extruded at 60°C through a 2 μm polycarbonate membrane (Nuclepore) and, after cooling to room temperature, it was concentrated to 30 mg/ml by microfiltration using a 0.22 μm microfilter (Millipore).

To the concentrated liposome solution, there was added 1 l of an aqueous solution containing 1040 g of (S)-N,N'-bis[2-hydroxy-1-(hydroxymethyl)-ethyl]-2,4,6-triiodo-5-lactamido-isophthalamide (Iopamidol, an X-ray contrast agent produced by BRACCO INDUSTRIA CHIMICA, Milano) i.e. 520 g/l of covalent iodine at 60°C. The resulting mixture (2 l) had an iodine concentration of 260 g/l and was incubated for about 30 min at 60°C, after which time the iodine concentration outside and inside the liposome core had equalized. The re-

sulting preparation was concentrated to 30 g lipids/l (Preparation A).

Preparation A was analyzed for lipids and encapsulated iodine. For this, an aliquot (1 ml) was dialyzed against saline (NaCl 0.9% in water) until all iopamidol outside the liposomes vesicles had been removed (about 24 hours with 4 changes of the dialysis medium). The sample was then treated at 50°C with 1/10th of its volume of a 10% sodium dodecyl sulfate solution in water and the liberated Iopamidol was measured spectrophotometrically at 260 nm. The corresponding amount of lipids was determined by counting with a scintillation counter using the residual radioactivity of the tri-palmitin tracer. The results of the foregoing analysis showed that the encapsulation capacity measured as the iodine-to-lipid ratio (I/L) was consistently in the range of 3 to 5 mg (or more) of entrapped iodine per mg lipid, which means that the average internal captured volume of the liposome vesicles (calculated on the basis of an iodine concentration of 260 mg/ml) was about 12-19 µl/mg of lipid (or more).

Part of the preparation A of contrast agent-loaded liposomes of this example was diafiltered against buffered saline (0.9% NaCl, 10 mM Tris.HCl, pH 7.2) containing Na₂Ca EDTA (0.9 mM) using a 0.22 µm membrane (Millipore). The resulting preparation (Prep B) as well as preparation A were usable directly for injection into the bloodstream of experimental animals, both of them providing X-ray opacification of blood vessels and organs (e.g. liver and spleen) with extremely favorable results.

When, in the foregoing example, the Iopamidol was replaced by Iomeprol (N,N'-bis(2,3-dihydroxypropyl)-2,4,6-triiodo-5-glycolamido-isophtalimide), another iodinated contrast agent from BRACCO INDUSTRIA CHIMICA (Milano), similar entrapping results were experienced. When in the foregoing example, the Iopamidol was replaced by B17500, an experimental non-ionic dimer made by BRACCO, I/L values in excess of 6, exceeding sometimes 7 were obtained. Similar results were observed with Iotrolan, a non-ionic dimer produced by SCHERING AG.

Example 2

REV liposomes were prepared according to the method of Szoka et al. (Proc. Natl. Acad. Sci. USA 75 (1978), 4194). Briefly, hydrogenated soy lecithin (Phospholipon 90H from NATTERMANN PHOSPHOLIPID GmbH, 912.2 mg) and DPPA (92.9 mg) were dissolved in 80 ml of a 1:1 mixture of chloroform and isopropylether. To this, were added 30 ml of distilled water and the mixture was emulsified by sonication (5 x 1 min.) using a Branson probe sonifier, while maintaining the temperature at 45°C. Then the emulsion was evaporated at 45°C under reduced pressure in a rotary evaporator. After the evaporation of residual solvents was complete, and a small amount of distilled water had been added, a suspension of REV liposomes with 33 mg lipid/ml and an average size of 0.4 μm was obtained. Iopamidol (1.4 g) was dissolved in 2 ml of the suspension and the solution was incubated for 1 hour at 80°C. I/L values (measured as described in Example 1) of 2.1 - 2.3 were obtained.

Lower entrapment yields were obtained when there was used, for comparison, a Iopamidol solution (30 ml, 260 mg iodine per ml) instead of pure water for initially emulsifying with the organic solution of lipids.

These experiments were repeated with REV liposomes prepared using dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidyl glycerol (DPPG) (molar ratio 9/1) with distilled water as the aqueous phase and then incubated with sodium diatrizoate (215 or 21.5 mg iodine/ml) for 20 min. at 60°C. For comparison, REV liposomes were also prepared with the same phospholipids using as the initial aqueous phase sodium diatrizoate solutions (215 and 21.5 mg iodine per ml, respectively) instead of distilled water. Although entrapment yields of the same order of magnitude were obtained with the two approaches i.e. I/L values of about 1, resp. 0.2 at 215, resp. 21.5 mg iodine/ml, the technique starting with empty liposomes gave still better results.

Example 3

Liposomes of the SUV type were obtained by sonicating for 15 min. at 60°C, using a Branson probe sonifier, a suspension of MLV liposomes prepared in distilled water as described in Example 1. The supernatant obtained after centrifugation for 10 min. at 10'000 g was incubated with Iopamidol (final concentration 260 mg iodine/ml) for 20 min. at 60°C. An I/L value (measured as described in Example 1) of 0.16-0.17 mg iodine per mg lipid was obtained, corresponding to a captured volume of 0.6 µl/mg lipid.

Example 4

MLV liposomes were prepared in distilled water as described in Example 1. Prior to extrusion, the liposome suspension was repeatedly frozen (at -75°C) and thawed (at 40°C) four times according to the method of Mayer et al. (Biochim. Biophys. Acta 817 (1985), 193). Both extruded (2 µm) and non-extruded liposome suspensions were prepared and incubated at 60°C for 30 min. with a Iopamidol solution (260 mg iodine/ml). Extruded liposomes gave an I/L value of 5, whereas non-extruded liposomes gave an I/L value of 6.3. When, in a variant, extrusion was performed below the lipids transition temperature, e.g. at from room temperature to 50°C, higher entrapment yields (I/L = 8 or more) were recorded.

Example 5

Influence of temperature.

MLV liposomes were prepared in distilled water as described in Example 1 at various temperatures, i.e. 55, 60, 65, 70, 80°C. They were incubated with Iopamidol (final concentration 260 mg iodine/ml) at 60°C for 30 min. The following I/L values were obtained :

Temperature of liposome
formation (°C)

I/L
mg iodine/mg lipid

55	4.2
60	4.9
65	4.5
70	3.8
80	3.1

It can be seen that the optimal temperature for liposome formation is 60°C, i.e. 6° above the transition temperature of the lipid mixture used (Phospholipon 100H and DPPA in a 9:1 molar ratio).

The influence of the temperature of incubation was determined as follows : MLV liposomes were prepared in distilled water at 60°C as described in Example 1. Aliquots were then incubated at various temperatures with a Iopamidol solution (final concentration 260 mg iodine/ml). The following I/L values were obtained :

Temperature of incubation (°C)	I/L mg iodine/mg lipid
40	3.0
50	3.4
55	4.7
60	4.9
65	4.1
80	3.9

The optimal temperature for incubation is in the range 55-60°C i.e. 1 to 6° above the transition temperature of the mixture of lipids used.

Example 6

MLV liposomes were prepared in distilled water at 60°C like in Example 1 with various lipid concentrations, then they were incubated for 30 min. at 60°C with a iopamidol solution (260 mg iodine/ml) as described in Example 1. Aliquots were brought to 130°C for various time periods (see below), then rapidly cooled to room temperature. The following I/L values were measured :

Duration of incubation at 130°C (min.)	I/L mg iodine/mg lipid
1	3.9
2	3.8
4	3.8
6	3.7
8	3.8
10	3.6

It can be concluded that the liposomes of the invention are not altered with regard to their loading capacity by exposure to sterilizing temperatures.

Example 7

Influence of lipid concentration.

MLV liposomes were prepared at 60°C in distilled water like in Example 1 using various lipid concentrations. Then they were incubated with a iopamidol solution (260 mg iodine/ml) as described in Example 1. The following I/L values were obtained :

Lipid concentration at formation (mg/ml)	I/L mg iodine / mg lipid
2.5	3.8
5.0	3.6
10.0	3.5
25.0	2.3
50.0	1.9

The best results are obtained at lipid concentrations of 2.5 - 10 mg/ml.

MLV liposomes were prepared in distilled water at 60°C at a lipid concentration of 5 mg/ml. They were concentrated (between 5 and 35 mg lipid/ml) then incubated with a iopamidol solution (260 mg iodine/ml) as described in Example 1. The following I/L values were obtained :

Lipid concentration during incubation (mg/ml)	I/L mg iodine per mg lipid
5	3.6
9	3.8
14	3.9
18	4.0
25	3.7
35	3.7

There is therefore no influence of the lipid concentration during incubation with Iopamidol on the trapping capacity.

Example 8

MLV liposomes were prepared in distilled water, extruded through a 2 μ m membrane and concentrated to 35 mg lipid/ml as described in Example 1. To 1 ml aliquots of the concentrated liposome suspension (but 3 ml in the case of Prep. A) were added 1 ml aliquots of the following solutions:

Prep. A : Gd-DTPA (117 mM) labeled with a trace amount of ^{153}Gd .

Prep. B : a 4% lidocaine HCl solution in water adjusted to pH 7.2 with NaOH.

Prep. C : a sodium diatrizoate solution (215 mg iodine/ml).

Prep. D : a cis-platin solution in distilled water (10 mg/ml).

Prep. E : an aqueous insulin solution (20 mg/ml) with pH adjusted to 7.5.

Incubations were carried out at 80°C (Prep. A, Prep. B and Prep. C) or 60°C (Prep. D and Prep. E) during 30 min. The entrapped compounds were determined after dialysis by radioactive counting (Prep. A), HPLC (Prep. B), spectrophotometrically (Prep. C), atomic absorption (Prep. D). For Prep. E the non-entrapped insulin was removed by column chromatography on DEAE-A-50 Sephadex and the amount of entrapped material was measured by protein analysis (Method of Lowry). The following loadings and corresponding captured volumes were obtained :

Sample	Loading	Captured volume
Prep. A:	0.25 - 0.35 μ mol Gd/mg lipid	8.5 - 12 μ l/mg lipid
Prep. B:	0.35 μ mol lidocaine/mg lipid	5 μ l/ml lipid
Prep. C:	0.8 mg iodine / mg lipid	3.5 μ l/mg lipid
Prep. D:	5.9 μ g cis platin/mg lipid	1 μ l/mg lipid
Prep. E:	0.18 mg insulin/mg lipid	18 μ l/mg lipid

High captured volumes were observed for all products tested. Prep. A was repeated replacing Gd-DTPA by Gd-BOPTA meglumine salt, a new contrast agent for MRI (code B-19030; formula: 3-phenylmethoxy-2-N[2'-N'-(2''-N'''-bis-(carboxymethyl)-aminoethyl)-N'-(carboxymethyl)-aminoethyl]-N-(carboxymethyl)-aminopropionic acid) under development at BRACCO and similar results were obtained.

Example 9

Influence of the lipid composition.

Various phospholipid mixtures were evaluated in a series of experiments carried out as described in Example 1. The following I/L values were obtained :

Lipid composition (molar ratio)

I/L

Phospholipon 100H/DPPA.Na ₂ (9:9/0.1)	3.0
Phospholipon 100H/DPPA.Na ₂ (9.5/0.5)	3.7
Phospholipon 100H/DPPA.Na ₂ (9.25/0.75)	4.2
Phospholipon 100H/DPPA.Na ₂ (9/1)	4.9
Phospholipon 100H/DPPG (9/1)	4.8
Phospholipon 100H/Cholesterol/DPPA.Na ₂ (4.5/4.5/1) ^a	1.3
Phospholipon 100H/Cholesterol/DPPA.Na ₂ (6.75/2.23/1) ^b	2.2
Phospholipon 100H	1.4
Phospholipon 100H/Stearylamine (9/1)	1.7
DPPC/DPPA.Na ₂ (9/1) ^c	4.0
DPPC/DMPC/DPPA.Na ₂ (4.5/4.5/1) ^d	3.6
Phospholipon 100H/DCP.Na (9/1)	3.0
Phospholipon 90H/DSPA.Na ₂ (9/1)	

Legend: DPPG : dipalmitoyl phosphatidylglycerol sodium salt

DPPC : dipalmitoyl phosphatidyl choline

DMPC : dimyristoyl phosphatidyl choline

DCP.Na : dicetylphosphate sodium salt

The liposomes were prepared at the temperatures:

a: 40°C.

b: 50°C.

c: 50°C (i.e. 6°C above the transition temperature of the mixture of phospholipids).

d: 40°C (i.e. 4°C above the transition temperature of the mixture).

Example 10

MLV liposomes were prepared in distilled water as described in Example 1. After extrusion and concentration they were incubated with various concentrations of Iopamidol, in the absence (Series A) or presence (Series B) of NaCl. In the experiments of Series C, MLV liposomes were prepared directly in the iopamidol solution in the presence of various concentrations of NaCl. The following I/L values were obtained :

Iopamidol conc. (mg I ₂ per ml)	NaCl (mM)	I/L (mg I ₂ per mg lipid)	capt.vol (μl/mg)
Series A			
100	0	1.7	17
200	0	2.8	14
260	0	3.5	14
300	0	4.0	13
370	0	4.4	12
Series B			
215	56	2.1	10
215	565	0.7	3
Series C			
215	56	1.8	8
215	565	0.15	0.7

Thus increasing iopamidol concentrations resulted into increased loading, with no major impact on the captured volume (Series A). The presence of salt reduces the loading as well as the captured volume (Series B). Nevertheless higher loadings are achieved with the technique of the invention compared to the classical MLV technique (Series C).

Example 11

MLV liposomes were prepared in various aqueous solutions (instead of distilled water) at 60°C, then after extrusion and concentration, they were incubated for 30 min. at 60°C with a iopamidol solution (260 mg iodine/ml) (see Example 1). The following I/L values were obtained :

Medium used for the formation of MLV's	I/L (mg I ₂ /mg lipids)
---	---------------------------------------

Distilled water (as reference)	4.2 - 4.4
10 mM Tris/HCl pH 7.2, 0.9 mM EDTA	2.9 - 3.1
6 mM NaCl	3.0 - 3.3
56 mM NaCl	2.2 - 2.4
560 mM NaCl	0.7 - 0.8
146 mM trehalose	2.1 - 2.3
274 mM mannitol	1.5 - 1.8
iomeprol solution (260 mg iodine/ml)	0.7
(calc. as iopamidol)	

As seen in these experiments, a decrease in the entrapment of Iopamidol is observed in all cases when the vesicles are formed in a medium containing already a solute. The presence of ionic species such as NaCl at ionic strengths above 0.1 or of non electrolytes at Osmolalities of more than 200 mOsm/kg are particularly detrimental.

CLAIMS

1. A method for loading liposome vesicles suspended in an aqueous carrier liquid with substances to be encapsulated, said liposome vesicles consisting of a core filled with an aqueous liquid phase surrounded by one or more membranes of film-forming components including lamellar phospholipids and, optionally, non-phospholipid amphipatic compounds, which comprises introducing and distributing homogeneously said substances to be encapsulated into said carrier liquid and incubating at a temperature above the membrane lipid transition temperature T_C for a period of time, whereby said substances to be encapsulated will penetrate into the core of the vesicles by trans-membrane permeation, characterized in that the osmolality of the liquid phase contained in the vesicle internal core phase before loading is not above 200 mOsm/kg.

2. The method of claim 1, in which a mixture of liposome forming lipids, at least one component of which is ionically charged, is placed in contact with an aqueous liquid phase for a time sufficient to cause said lipid to become hydrated and lamellarized, characterized in that said contacting is effected in a step in which one part by weight of said lipid mixture in comminuted form is introduced and homogeneously distributed, with or without agitation, into at least 20 parts of said aqueous liquid phase, and in that the temperature of said liquid phase is kept during this step within a range of a few °C above the transition temperature (T_C) of said hydrated form of the lipid, whereby liposome vesicles will form with a high proportion of the liquid phase encapsulated therein and with enhanced transmembrane loading capacity.

3. The method of claim 1, in which the aqueous liquid that fills the core of the liposome vesicles also forms the aqueous carrier liquid in which the liposomes are suspended.

4. The method of claim 1, in which said substance to be encapsulated is ionic or non-ionic

5. The method of claim 1, in which incubation is allowed to proceed by heating the liposome dispersion to a temperature between T_C and about 150°C for a period of time until the concentrations of the dissolved substance in the carrier phase outside the liposomes and inside the core thereof are substantially balanced.

6. The method of claim 5, in which the heating temperature is above 100°C and the heating time is sufficient to ensure sterilization of the liposomes.

7. The method of claim 2, in which the ratio of the volume captured in the liposomes internal core to the weight of the lipids making the liposome vesicles walls is not lower than 5 $\mu\text{l}/\text{mg}$.

8. The method of claim 2, in which the step of distributing the lipids into the carrier phase is effected under agitation, the average size of the liposomic vesicles thus obtained being inversely proportional to the degree of said agitation.

9. The method of claim 1, in which the aqueous liquid where the liposomes are formed is pure water, whereby the liposomes that form are empty liposomes only containing water.

10. The method of claim 2, in which the volume captured in the liposomes internal core is increased by repeated freeze-and-thaw steps and/or dehydration/rehydration steps.

11. The method of claim 2, in which the liposome dispersion is forced through a calibrated porous membrane to homogenize the size distribution of the vesicles.

12. The method of claim 1, in which the substances to be encapsulated are selected from drugs in general and injectable diagnostic reagents.

13. The method of claim 11, in which said diagnostic reagent is an organic iodinated X-ray contrast agent which is loaded into the liposomes during incubation to the extent that the loading efficiency expressed in mg of iodine per mg of lipids (I/L) may reach values above 3.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 91/02377

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁴		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 A61K9/127		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁵		
Classification System	Classification Symbols	
Int.Cl. 5	A61K	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁶		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁷		
Category ⁸	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	EP,A,0 346 472 (TERUMO K.K.) 20 December 1989	1-5,7-11
Y	see pages 8,9, example and comparative example 1 ---	12,13
X	FR,A,2 298 318 (TANABE SEIYAKU CO.,LTD) 20 August 1976 see page 7, line 4 - line 27 ---	1,3-11
X	WO,A,8 905 636 (THE LIPOSOME COMPANY, INC.) 29 June 1989 see page 18 - page 19; examples 14,15 ---	1-5,7-11
Y	WO,A,8 809 165 (BRACCO INDUSTRIA CHIMICA S.P.A.) 1 December 1988 see the claims --- -/-	12,13
<p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not to conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
16 MARCH 1992	30. 03. 92	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	BENZ K.F. <i>Benz</i>	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)	
Category *	Citation of Document, with indication, where appropriate, of the relevant passages
A	<p>WORLD PATENTS INDEX LATEST Derwent Publications Ltd., London, GB; AN 85-052231 (09) & JP,A,60 007 934 (DAIICHI SEIYAKU K.K.) 16 November 1985 see abstract</p>
	1-13

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. EP**

9102377
SA 54248

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 16/03/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0346472	20-12-89	JP-A- 63211222	02-09-88
		AU-B- 607978	21-03-91
		AU-A- 1364788	26-09-88
		WO-A- 8806436	07-09-88
FR-A-2298318	20-08-76	JP-C- 1018539	28-10-80
		JP-A- 51086117	28-07-76
		JP-B- 55008488	04-03-80
		DE-A, B, C 2601207	29-07-76
		GB-A- 1487989	05-10-77
		US-A- 4016100	05-04-77
WO-A-8905636	29-06-89	US-A- 4963297	16-10-90
		EP-A- 0393145	24-10-90
WO-A-8809165	01-12-88	CH-A- 672733	29-12-89
		AU-B- 612371	11-07-91
		AU-A- 1805488	21-12-88
		EP-A- 0314764	10-05-89
		JP-T- 2500192	25-01-90
		ZA-A- 8803449	16-11-88